Oral Microbiology Immunology 2002: 17: 60–64
Printed in Denmark. All rights reserved

Oral Microbiology and Immunology
ISSN 0902-0055

Candida attachment to oral epithelium

L. Vitkov[1,2], W. D. Krautgartner[2], M. Hannig[3], R. Weitgasser[4], W. Stoiber[2]

[1]Private Practice, Salzburg and [2]Department of Electron Microscopy, Light Microscopy and Digital Image Acquisition, University of Salzburg, Austria, [3]Department of Operative Dentistry and Periodontology, University of Freiburg, Germany, [4]First Medical Department, St. Johanns Spital Landeskliniken, Salzburg, Austria

Vitkov L, Krautgartner WD, Hannig M, Weitgasser R, Stoiber W. *Candida* attachment to oral epithelium.
Oral Microbiol Immunol 2002: 17: 60–64. © Munksgaard, 2002.

Inflamed oral mucosa biopsies from patients with thrush and high candidal density were observed in a transmission electron microscope (TEM) using ultrahistochemical staining with ruthenium red for glycocalyx visualization. Fimbriae comprising the glycocalyx and enabling yeast adhesion to epithelial cells were clearly visualized by ruthenium red. All internalized portions of the yeast walls were devoid of glycocalyx, indicating that the growing tips of the hyphae mechanically penetrated the host cells. The attachment of *Candida* occurred in two ways: by fimbria-mediated adhesion enabling colonization of the epithelial surface, and by invasion of the superficial epithelial cells via hyphae. As the interaction between adhesin receptors and adhesins stimulates the production of proinflammatory cytokines, *Candida* adhesion itself is assumed to induce mucosal inflammation.

Key words: adhesins; *Candida*; fimbriae; oral epithelium; transmission electron microscopy

L. Vitkov, Department of Electron Microscopy, Institute of Zoology, University of Salzburg, Hellbrunnerstraße 34, A-5020 Salzburg, Austria
Accepted for publication June 28, 2001

The initial stage of epithelial colonization by *Candida* is thought to be the adherence of the yeast cell to the mucosal surface (16, 22). The ability of a yeast strain to adhere to human epithelial cells has been correlated with virulence in animal models (8, 22). Adhesion takes place by means of long, thin filamentous cell surface appendages termed fimbriae. These form an extracellular coat (glycocalyx). *Candida* can easily be induced to form fimbriae *in vitro* by addition of a high concentration of carbohydrates (6, 18, 24). Candidal fimbriae formed *in vitro* have been visualized in a transmission electron microscope (TEM) by ruthenium red staining (19), similarly to bacterial fimbriae (10). Although it is not possible to maintain such a permanently high concentration of carbohydrates in the oral cavity, other mechanisms could stimulate yeast fimbrial formation in oral *Candida*-associated diseases. Similarly, bacteria produce glycocalyx *in vitro* under the influence of high carbohydrate concentrations (5). Recently, electron microscope investigations have demonstrated bacterial attachment to the palatal epithelium in denture stomatitis occurring by fimbria-mediated adhesion (25). Elucidation of the nature of *Candida* attachment to the human oral epithelium in *Candida*-induced diseases will contribute to understanding of the pathogenicity of this yeast.

The aim of this work was to investigate, by electron microscopy, the attachment of *Candida* to the oral epithelium in patients with thrush.

Materials and methods

Thrush, as an exacerbation of previously known stomatitis, was diagnosed by routine oral inspections in six subjects with denture stomatitis and in two spray inhalers (Berodual-Dosier aerosol; Boehringer, Ingelheim, Germany; 3 times a day) with iatrogenic stomatitis of the soft palate. All patients were in the age bracket of 41–71 years (mean: 60 years). The spray inhalers were denture wearers without denture stomatitis and had an intake of 10 mg prednisolon weekly. The criteria suggested by Holmstrup and Axéll (15) were used for diagnosis of thrush. The study was approved by the local ethics committee, and informed written consent was obtained from all patients. The *Candida* density was estimated as described previously (27). In addition, samples for microbiological investigation were taken with swabs (Biotest Transportsystem, Biotest AG, Dreieich, Germany) from about 1 cm^2 of the mucosa, near to the place where the excision was to be made. Sampling took place between 0900 and 1200 h, at least 2 h after the patient's breakfast. For aerobic culturing, each swab was plated under aerobic conditions on Columbia sheep blood agar (Biotest AG), Columbia CNA agar (Becton Dickinson, Franklin Lakes, NJ) and MacConkey agar (Biotest AG), and incubated at 37°C for 18 h. For anaerobic culturing, the swabs were plated on Schaedler agar (Biotest AG) and Schaedler KV agar (Becton Dickinson), and incubated at 37°C for 7 days. Microbiological differentiation was performed as follows.

Viridans streptococci were identified by colony morphology, alpha-haemolysis, and use of a commercially available optochin test. Nonhaemolytic strepto-

cocci were identified by colony morphology, lack of haemolysis and the optochin test. Enterococci were identified by colony morphology and antibiotic susceptibility. Staphylococci were identified by colony morphology, colony colour, and testing of coagulase activity with Staphaurex (Abbott Laboratories, Abbott Park, IL). *Hemophilus* spp. were identified by colony morphology, gram staining, and biochemical identification procedures API NH (Biomerieux SA, Marcy l'Etoile, France). *Neisseria* spp. were identified by colony morphology, gram staining and positive oxidase reaction. Gram-negative bacilli (*Escherichia coli*, *Klebsiella*, *Enterobacter*, etc.) as well as *Lactobacillus casei* were identified by use of a biochemical identification system (Crystal System, Becton Dickinson, Franklin Lakes, NJ).

For semiquantitative estimation of the bacterial density, the following counting procedure was used.

- Negative bacterial growth was characterized by 0–4 colonies per agar plate.
- Low bacterial density was defined by the growth of at least five colonies per agar plate.
- Moderate bacterial density was defined by growth over half of the agar plate.
- High bacterial density was defined by confluent bacterial growth.

Biopsies (approx. 5 × 8 mm) were taken from the inflamed mucosa of each patient under regional block anaesthesia. Each biopsy was washed in 50 ml saline by gently swaying for 20 s and subsequently divided into two equal parts. The specimens were washed to remove all substances in contact with but not attached to the epithelial surface. One sample from each patient was fixed with 1.2% glutaraldehyde (buffered at pH 6.5 with 0.1 M sodium cacodylate) for 2 h at 4°C. Postfixation of these control samples was performed with 1% osmium tetroxide (buffered at pH 6.5 with 0.1 M sodium cacodylate) for 2 h at 4°C. The other sample from each patient was fixed and postfixed by the fixatives mentioned above with addition of 0.05% ruthenium red in each case. All specimens were routinely embedded in Epon 812. Ultrathin sections were cut on an ultramicrotome (Reichert Ultracut S, Optische Werke C. Reichert, Vienna, Austria), contrasted with LKB 2168 Ultrostainer (LKB Produkter AB, Bromma, Sweden) and examined in a transmission electron microscope LEO EM 910 (LEO Elektronenmikroskopie Ltd, Oberkochen, Germany) operating at 80 kV.

Results

Results of the microbiological examination are summarized in Table 1. The predominant yeast species found in the samples was *Candida albicans*. Certain differences concerning the bacterial flora were detected between individuals. The normal oral bacteria were represented by viridans streptococci and *Neisseria* spp. *Staphylococcus epidermidis* was found in 50% of the patients, and anaerobes were found in 12%.

Electron-microscopically, numerous yeasts were identified intra- and extracellularly (Fig. 1). Different *Candida* cell numbers were observed between and within individual samples. A fairly uniform number of circular or ovoid transverse sections of the fungi were present in the ultra-thin sections, making it difficult to distinguish between sections through spherical/ovoid blastospores and tubular hyphae. The cell walls and cytoplasmic contents were similar in all circular, ovoid and tubular yeast sections. The yeast was predominantly located on and/or within desquamated and loose epithelial cells (Fig. 1).

The samples fixed in the absence of ruthenium red showed no (Fig. 2) or a rather faint layer exterior to the cell wall (Fig. 3). However, staining with ruthenium red revealed the existence of a thick layer of fibrils with high electron

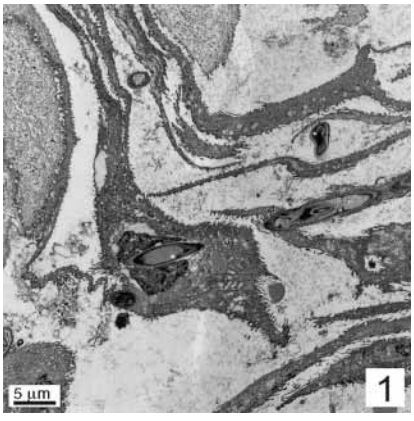

Fig. 1. Control staining, showing loose palatal epithelium with extra- and intracellular yeasts.

Table 1. Results of the microbiological investigation: yeast and bacterial species and density in patients 1–8

Patient	Age (yr)	Sex	*Candida* spp.	Viridans streptococci	*Neisseria* spp.	*Enterococcus faecalis*	*Staphylococcus epidermidis*	*Escherichia coli*	*Enterobacter aerogenes*	*Klebsiella oxytoca*	*Morganella morganii*	*Bacteroides* spp.
1	49	m	alb + spp ++			+++			+++			
2	61	m	alb +++	++			+++	+++		+++		
3	71	m	alb +++	++		++	++					
4	41	f	alb + spp ++	++	++							
5	60	m	alb +++	++			+	+++				
6	64	f	alb +++			+						
7	68	m	alb +++	++		++	++					+
8	66	m	alb +++	+++	++					++	++	

+ = low yeast or bacterial density; ++ = moderate density; +++ = high density; alb = *Candida albicans*; spp = *Candida* non-*albicans* species.

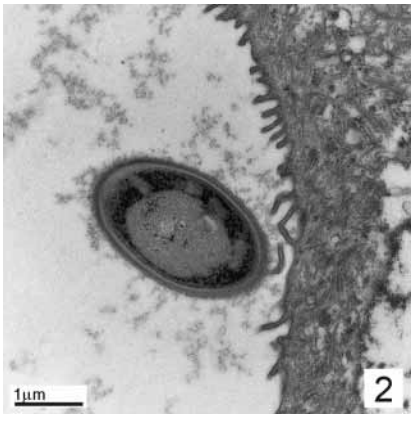

Fig. 2. Control staining, showing absence of a layer exterior to the yeast cell wall, and thickening of the epithelial cytoplasm around the yeast.

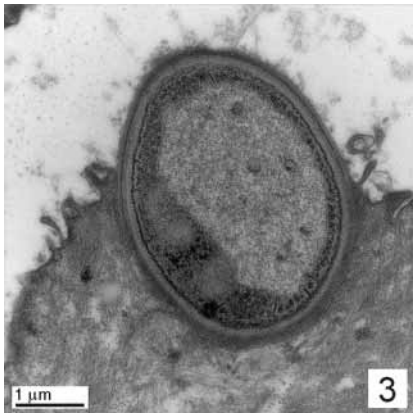

Fig. 3. Control staining, showing a faint layer exterior to the yeast cell wall outside the epithelial cell.

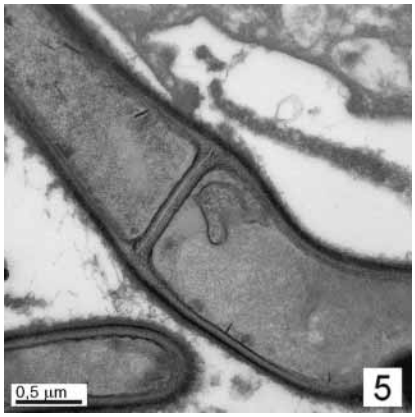

Fig. 5. Ruthenium red staining, showing formation of a dividing wall between two true hyphae.

density exterior to the cell wall (Figs 4, 8 and 9). The TEM analysis showed yeast adhesion to the epithelium and internalization in all biopsy samples. The fimbriae were not always easily distinguishable (Figs 4 and 7) because of their high electron density, but in some cases they were clearly distinguishable on the eukaryotic cell membrane (Figs 5, 6 and 8). All parts of the internalized yeast walls were devoid of glycocalyx (Fig. 6).

In cases where hyphal tip growth towards the loosened epithelial cell was observed, a deformation of the epithelial membrane and cytoskeleton and the formation of an epithelial cell invagination around the depressing hyphal tip were seen (Figs 7 and 9).

All areas where bacteria were observed were located superficially scattered on the epithelial surface. High ultrastructural diversity (cocci, coccus-like and rod-shaped bacteria) was observed in the bacteria adhering to the outer surface of the epithelial cell membranes (Fig. 7) or to other bacteria (Fig. 9). In addition, a certain morphological similarity between bacterial (Fig. 10) and yeast (Fig. 8) glycocalyces, both consisting of fimbriae, was evident.

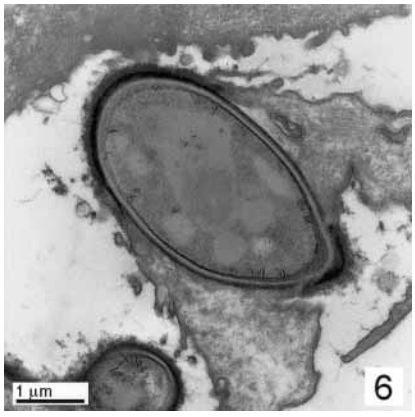

Fig. 6. Ruthenium red staining, showing an epithelial cell penetrated by a yeast, with glycocalyx formation on the yeast cell wall only outside the epithelial cell.

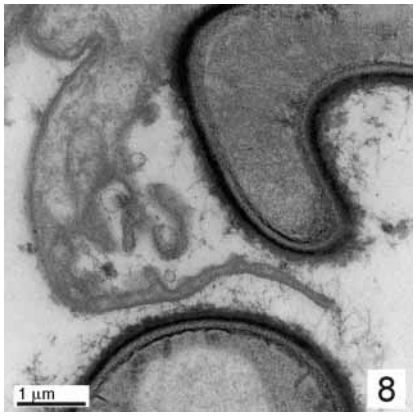

Fig. 8. Ruthenium red staining, showing separate fimbriae adhering to the epithelial cell membrane.

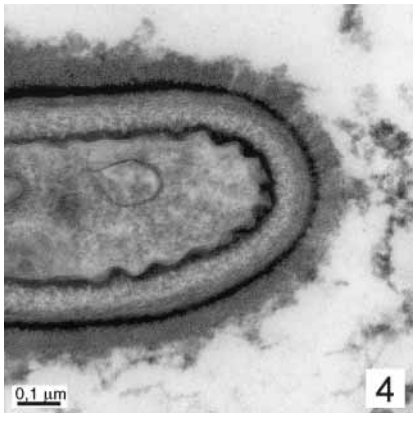

Fig. 4. Ruthenium red staining, showing a large yeast glycocalyx with very electron-dense contrasting exterior to the cell wall.

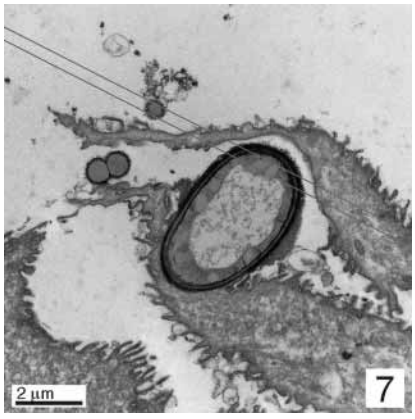

Fig. 7. Ruthenium red staining of a longitudinal section through an epithelial cell invagination containing a growing hypha. The epithelial cell surface is distinctly deformed. The two lines mark the boundary of a cross-section as demonstrated below in Fig. 9. Diplococcus or dividing cocci can be seen adhering to the epithelial cell membrane.

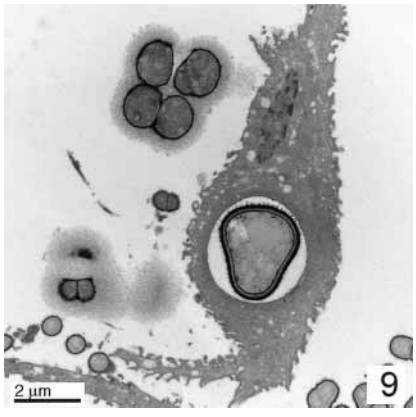

Fig. 9. Ruthenium red staining of a cross-section through a shallow epithelial cell invagination containing a growing hypha. The very electron-dense glycocalyx contrasted by ruthenium red provides unequivocal evidence of the extracellular position of the fungus, since ruthenium red is unable to penetrate the epithelial cell membrane (8). The separate fimbriae adhering to the epithelial cell membrane are clearly distinguishable. Bacteria adhered by glycocalyces with different largeness.

Discussion

The present study provides, for the first time, ultrastructural evidence of fimbriae-mediated *Candida* adhesion to human oral epithelium in a *Candida*-in-

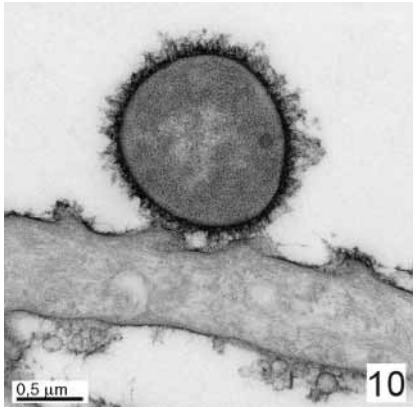

Fig. 10. Ruthenium red staining, showing a bacterium adhering by fimbriae to the epithelial cell membrane.

duced disease. The adhesins recognize and bind to particular receptors expressed on the eukaryotic cell membrane. Morphologically, the *Candida* adhesins are integrated into fimbriae. The major structural unit of the *C. albicans* fimbriae consists of 80–85% carbohydrate (primarily D-mannose) and 10–15% protein, so that the fimbriae are biochemically analogous to the bacterial fimbriae (29). Some morphological similarity between the fimbriae of bacteria and *Candida* is also indicated in the present TEM micrographs.

As carbohydrates are not stainable by routine contrasting substances, glycocalyx cannot be easily monitored in TEM, unless special staining techniques are used. The ability of cationic ruthenium red to enhance staining of acidic glycosylated structures at the ultrastructural level is widely used for the visualization of such structures (9, 10,14, 25). The extracellular fungi stained by ruthenium red displayed a thick glycocalyx, which was seen in some cases in control staining as a faint coating, similarly to the floccular layer described in oral candidiasis (21) and above the denture plaque (26). Electron-microscopically, the glycocalyx of the oral yeasts showed the same base characteristics as the glycocalyx of *Candida* grown *in vitro* with addition of 0.5 M sucrose (18) or 0.5 M galactose (6). The partial visualization of *Candida* glycocalyx in non-ruthenium-red-stained specimens could result from staining the proteinaceous part of the fimbriae or proteins adsorbed on them (21).

Epithelial cells cannot be penetrated by blastospores, but only by the growing hyphal tips (16). The attachment via glycocalyx to some surfaces provides anchorage for the hyphae and allows growth through the epithelial tissue. Consequently, penetration can occur only if anchorage by adhesion has taken place in advance.

An adhesin constituent, the supernatant of the mannoprotein fraction (MP-F2), elicits a greatly delayed type hypersensitivity reaction, Th1 and Th2 cytokine production and anticandidal protection in mice (20). Another glycocalyx constituent, beta-1,2-linked oligomannoside residues of *C. albicans,* acts as an adhesin for macrophages and stimulates these cells to undergo cytokine production (11). *C. albicans* mannan stimulates the production of IL-2, IL-4 and gamma interferon by mouse splenocytes (17). In addition, the mannose receptors of macrophages are involved in cytokine IL-1β, IL-6 and granulocyte-macrophage colony-stimulating factor responses (28). As the interaction between adhesin receptors and adhesins stimulates the production of proinflammatory cytokines, the fimbria-mediated adhesion of *Candida* could consequently be regarded as a primary inducer of the *Candida*-induced inflammation, which is further enhanced by *Candida* toxins (4, 7) and enzymes (4, 19).

C. albicans has the ability to adhere *in vitro* to the host cell surface receptors via a number of adhesins (30). The fimbriae of *Candida* possibly interact with the glycosphingolipid receptors via the carbohydrate portion of the receptors, displayed on the surface of human buccal epithelial cells (30). In addition, purified adhesins of *C. albicans* bind to glycolipids carrying the H blood group antigen (3). Among possible candidates for *Candida* receptors on epithelial cells are the ABO and Lewis blood group antigens (overall H and Lewis blood group antigens), all of which posses residues of L-fucose (3, 23). An association was demonstrated between blood group O, with nonsecretion of blood group antigens, and *Candida* carriage (1, 2). It is very likely that the salivary blood group antigens bind to the *Candida* adhesins and hinder the adhesion of the yeasts to the cytoplasmic membrane surfaces. Therefore, host susceptibility to the opportunistic pathogen *Candida* might largely be determined by the expression of adhesin receptors (blood group antigens) on the cytoplasmic membrane surfaces and particularly by nonsecretion of blood group antigens in saliva.

Antiserum against adhesins protects against disseminated candidiasis in animal models (12), and a vaccine against adhesin in animal models induces production of a monoclonal antibody protecting against candidiasis (13). It should be possible to develop an immunization protocol for *Candida* adhesins that leads to memory B-cell responses and long-term immunity against various forms of candidiasis (13).

The ultrastructural evidence presented for fimbria-mediated adhesion in thrush highlights the role of adhesion as a triggering factor in *Candida*-induced oral pathology.

Acknowledgments

The authors thank Mrs Adelina Vitkov for secretarial assistance. This work was supported by the Medical Association of Salzburg, Austria and the Medical Research Society of Salzburg, Austria.

References

1. Ben-Aryeh H, Blumfield E, Szargel R, Laufer D, Berdicevsky I. Oral *Candida* carriage and blood group antigen secretor status. Mycoses 1995: **38**: 355–358.
2. Burford-Mason AP, Willoughby JM, Weber JC. Association between gastrointestinal tract carriage of *Candida*, blood group O, and nonsecretion of blood group antigens in patients with peptic ulcer. Dig Dis Sci 1993: **38**: 1453–1458.
3. Cameron BJ, Douglas LJ. Blood group glycolipids as epithelial cell receptors for *Candida albicans*. Infect Immun 1996: **64**: 891–896.
4. Chattaway FW, Odds FC, Barlow AJ. An examination of the production of hydrolytic enzymes and toxins by pathogenic strains of *Candida albicans*. J Gen Microbiol 1971: **67**: 255–263.
5. Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Immun 1982: **37**: 318–326.
6. Critchley IA, Douglas LJ. Role of glycosides as epithelial cell receptors for *Candida albicans*. J Gen Microbiol 1987: **133**: 637–343.
7. Cutler JE, Friedman L, Milner KC. Biological and chemical characterization of toxic substances from *Candida albicans*. Infect Immun 1972: **6**: 616–627.
8. Diez-Orejas R, Molero G, Rios-Serrano I, Vazquez A, Gil C, Nombela C et al. Low virulence of a morphological *Candida albicans* mutant. FEMS Microbiol Lett 1999: **176**: 311–319.

9. Duncan MJ, Nakao S, Skobe Z, Xie H. Interactions of *Porphyromonas gingivalis* with epithelial cells. Infect Immun 1993: **61**: 2260–2265.
10. Fassel TA, Edmiston, CE Jr. Ruthenium red and the bacterial glycocalyx. Biotech Histochem 1999: **74**: 194–212.
11. Fradin C, Poulain D, Jouault T. Beta-1,2-linked oligomannosides from *Candida albicans* bind to a 32-kilodalton macrophage membrane protein homologous to the mammalian lectin galectin-3. Infect Immun 2000: **68**: 4391–4398.
12. Han Y, Cutler JE. Antibody response that protects against disseminated candidiasis. Infect Immun 1995: **63**: 2714–2719.
13. Han Y, Riesselman MH, Cutler JE. Protection against candidiasis by an immunoglobulin G3 (IgG3) monoclonal antibody specific for the same mannotriose as an IgM protective antibody. Infect Immun 2000: **68**: 1649–1654.
14. Hasty DL, Ofek I, Courtney HS, Doyle RJ. Multiple adhesins of streptococci. Infect Immun 1992: **60**: 2147–2152.
15. Holmstrup P, Axéll T. Classification and clinical manifestations of oral yeast infections. Acta Odontol Scand 1990: **48**: 57–59.
16. Howlett JA, Squier A. *Candida albicans* ultrastructure: colonization and invasion of oral epithelium. Infect Immun 1980: **29**: 252–260.
17. Li SP, Lee S, Domer JE. Alterations in frequency of interleukin-2 (IL-2)-, gamma interferon-, or IL-4-secreting splenocytes induced by *Candida albicans* mannan and/or monophosphoryl lipid A. Infect Immun 1998: **66**: 1392–1399.
18. McCourtie J, Douglas LJ. Relationship between cell surface composition of *Candida albicans* and adherence to acrylic after growth on different carbon sources. Infect Immun 1981: **32**: 1234–1241.
19. McMullan-Vogel CG, Jude HD, Ollert MW, Vogel CW. Related serotype distribution and secretory acid proteinase activity of *Candida albicans* isolated from the oral mucosa of patients with denture stomatitis. Oral Microbiol Immunol 1999: **14**: 183–189.
20. Mencacci A, Torosantucci A, Spaccapelo R, Romani L, Bistoni F, Cassone A. A mannoprotein constituent of *Candida albicans* that elicits different levels of delayed-type hypersensitivity, cytokine production, and anticandidal protection in mice. Infect Immun 1994: **62**: 5353–5360.
21. Montes LF, Wilborn WH. Ultrastructural features of host-parasite relationship in oral candidiasis. J Bacteriol 1968: **96**: 1349–1356.
22. Ray TL, Digre KB, Payne CD. Adherence of *Candida* species to human epidermal corneocytes and buccal mucosal cells: correlation with cutaneous pathogenicity. J Invest Dermatol 1984: **83**: 37–41.
23. Tosh FD, Douglas LJ. Characterization of a fucoside-binding adhesin of *Candida albicans*. Infect Immun 1992: **60**: 4734–4739.
24. Tronchin G, Bouchara J, Robert R, Senet J. Adherence of *Candida albicans* germ tubes to plastic: ultrastructural and maolerular studies of fibrillar adhesins. Infect Immun 1988: **56**: 1987–1993.
25. Vitkov L, Krautgartner WD, Hannig M, Fuchs K. Bacterial attachment to oral epithelium. Microsc Microanal 2000: **6** (Suppl. 2): 670–671.
26. Vitkov L, Krautgartner WD, Simonsberger P, Lugstein A. Die Morphologie der Prothesenplaque bei Prothesenstomatitis. Deut Zahnärztl Z 1999: **54**: 489–493.
27. Vitkov L, Weitgasser R, Lugstein A, Noack MJ, Fuchs K, Krautgartner WD. Glycaemic disorders in denture stomatitis. J Oral Pathol Medical 1999: **28**: 406–409.
28. Yamamoto Y, Klein TW, Friedman H. Involvement of mannose receptor in cytokine interleukin-1beta (IL- 1beta), IL-6, and granulocyte-macrophage colony-stimulating factor responses, but not in chemokine macrophage inflammatory protein 1beta (MIP-1beta), MIP-2, and KC responses, caused by attachment of *Candida albicans* to macrophages. Infect Immun 1997: **65**: 1077–1082.
29. Yu L, Lee KK, Ens K, Doig PC, Carpenter MR, Staddon W, Hodges RS et al. Partial characterization of a *Candida albicans* fimbrial adhesin. Infect Immun 1994: **62**: 2834–2842.
30. Yu L, Lee KK, Sheth HB, Lane-Bell P, Srivastava G, Hindsgaul O, Paranchych W et al. Fimbria-mediated adherence of *Candida albicans* to glycosphingolipid receptors on human buccal epithelial cells. Infect Immun 1994: **62**: 2843–2848.